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P-Rex1 and Vav1 Cooperate in the Regulation of Formyl-Methionyl-Leucyl-Phenylalanine–Dependent Neutrophil Responses

Campbell D. Lawson,* Sarah Donald,* Karen E. Anderson,* Daniel T. Patton,† and Heidi C. E. Welch*

G protein-coupled receptor (GPCR) activation elicits neutrophil responses such as chemotaxis and reactive oxygen species (ROS) formation, which depend on the small G protein Rac and are essential for host defense. P-Rex and Vav are two families of guanine-nucleotide exchange factors (GEFs) for Rac, which are activated through distinct mechanisms but can both control GPCR-dependent neutrophil responses. It is currently unknown whether they play specific roles or whether they can compensate for each other in controlling these responses. In this study, we have assessed the function of neutrophils from mice deficient in P-Rex and/or Vav family GEFs. We found that both the P-Rex and the Vav family are important for LPS priming of ROS formation, whereas particle-induced ROS responses and cell spreading are controlled by the Vav family alone. Surprisingly, fMLF-stimulated ROS formation, adhesion, and chemotaxis were synergistically controlled by P-Rex1 and Vav1. These responses were more severely impaired in neutrophils lacking both P-Rex1 and Vav1 than those lacking the entire P-Rex family, the entire Vav family, or both P-Rex1 and Vav3. P-Rex1/Vav1 (P1V1) double-deficient cells also showed the strongest reduction in fMLF-stimulated activation of Rac1 and Rac2. This reduction in Rac activity may be sufficient to cause the defects observed in fMLF-stimulated P1V1 neutrophil responses. Additionally, Mac-1 surface expression was reduced in P1V1 cells, which might contribute further to defects in responses involving integrins, such as GPCR-stimulated adhesion and chemotaxis. We conclude that P-Rex1 and Vav1 together are the major fMLFR -dependent Dbl family Rac-GEFs in neutrophils and cooperate in the control of fMLF-stimulated neutrophil responses. ***The Journal of Immunology*****, 2011, 186: 000–000.**

The small G protein Rac is a key regulator of cytoskeletal structure (and hence cell shape, adhesion, motility, phagocytosis, and regulated secretion), reactive oxygen species (ROS) production, and gene expression (1, 2). Rac2 is the predominant isoform in human neutrophils, whereas Rac1 and Rac2 are expressed equally in mouse neutrophils (3). Rac is essential for neutrophil function. Loss of Rac2 activity in humans (D57N mutation) results in severe recurrent bacterial infections, and $Rac2^{D57N}$ neutrophils are largely unable to chemotax to sites of infection, produce ROS, or secrete granule proteins (4, 5). $Rac2^{-/-}$ mice cannot clear fungal infections (6), and, in $Rac2^{-/-}$ neutrophils, adhesion, chemotaxis (polarization and motility), ROS formation, phagocytosis, and degranulation are impaired (6–10). In contrast, conditional $Rac1^{-/-}$ neutrophils produce ROS normally and can move, but with poor directionality (11, 12). Both Rac1 and Rac2 are required for the killing of *Escherichia coli* (9).

While Rac1 and Rac2 are essential for neutrophil-mediated immune defense, dysregulated Rac activity contributes to neutrophil-dependent inflammatory disorders. In $Rac1^{-/-}$/$Rac2^{-/-}$ mice, neutrophil infiltration into infection-induced arthritic joints is reduced at the acute stage, and disease development is delayed (although it is exacerbated at later stages) (13). Similarly, tissue damage is reduced in $Rac2^{-/-}$ mice in an acute lung injury model, with Rac2 being required for neutrophil infiltration and the release of granule proteins into the lung (14).

Rac can be activated by at least 20 different guanine-nucleotide exchange factors (GEFs) and, in turn, active Rac can stimulate a wide range of target proteins. Although many GEFs show tissue-restricted distribution, several different GEF families are often expressed in one cell type at any given time. The general view is that a large number of Rac-GEFs are required to convey signaling specificity, such that different stimuli activate different GEFs, which then couple Rac to different downstream pathways (15). Two families of Dbl-type GEFs, P-Rex and Vav, are known to activate Rac in neutrophils. Both can signal in response to G protein-coupled receptor (GPCR) stimulation, although the Vav family is best characterized for signaling downstream of integrins, FcRs, and TLRs. P-Rex GEFs are directly targeted by Gβγ subunits of heterotrimeric G proteins and phosphatidylinositol 3,4,5-trisphosphate (PIP_3), a lipid second messenger that is generated by PI3K (16). Neutrophils express a Gβγ-dependent form of PI3K; hence, both signals for P-Rex activation (Gβγ and PIP_3) are produced upon

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Abbreviations used in this article: FRET, fluorescence resonance energy transfer; GEF, guanine-nucleotide exchange factor; GPCR, G protein-coupled receptor; P1, P-$Rex1^{-/-}$; P12, P-$Rex1^{-/-}$/P-$Rex2^{-/-}$; P1V1, P-$Rex1^{-/-}$/$Vav1^{-/-}$; P1V3, P-$Rex1^{-/-}$/$Vav3^{-/-}$; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; pRGD, poly (arginyl-glycyl-aspartic acid); ROS, reactive oxygen species; V123, $Vav1^{-/-}$/$Vav2^{-/-}$/$Vav3^{-/-}$; WT, wild-type.

GPCR stimulation. Vav activation is largely mediated through Src and Syk family protein tyrosine kinases (17–19). Work carried out with COS^{phox} cells (which stably express the components of the neutrophil NADPH oxidase) suggests that, upon GPCR stimulation, Vav1 may be activated further by interacting with p67^{phox} (20). Interestingly, overexpression of P-Rex1, but not Vav1, is sufficient to elicit a GPCR-dependent ROS response in this system (21).

Of the P-Rex family, only P-Rex1 is expressed in neutrophils (16, 22). P-Rex1$^{-/-}$ neutrophils have a partial reduction in GPCR-dependent Rac2 activation, whereas Rac1 activation is near normal (23). P-Rex1 deficiency leads to a strong defect in GPCR-dependent ROS production in LPS-primed neutrophils, but the response is less affected in unprimed or TNF-primed cells (23). Chemotaxis of isolated neutrophils is only slightly reduced, with a mild defect in cell speed but normal polarization and directionality, although recruitment of neutrophils to inflammatory sites in vivo is substantially impaired (23, 24). Hence, P-Rex1 controls a subset of Rac-dependent neutrophil functions.

The predominant isoforms of the Vav family in mouse neutrophils are Vav1 and Vav3 (25). Similarly to P-Rex1, Vav1-deficient neutrophils have reduced GPCR-dependent ROS formation and a minor defect in chemotaxis (26). Integrin-dependent sustained adhesion and spreading are largely normal in cells deficient in Vav1 or Vav3 (25), but FcR-dependent phagocytosis and ROS production are impaired in Vav3$^{-/-}$ neutrophils (10). Neutrophils deficient in both isoforms (Vav1$^{-/-}$/Vav3$^{-/-}$) have defects in integrin-dependent sustained adhesion and spreading, as well as some (but not all) forms of complement-mediated phagocytosis, although their ability to chemotax toward GPCR ligands is unaffected (25, 27). FcR-dependent phagocytosis and ROS production are abolished in Vav1$^{-/-}$/Vav3$^{-/-}$ cells (10). Neutrophils lacking the entire Vav family (Vav1$^{-/-}$/Vav2$^{-/-}$/Vav3$^{-/-}$, or V123) have partially impaired TLR- and GPCR-dependent ROS formation, whereas integrin-dependent ROS formation is completely abrogated (28, 29). Hence, the members of the Vav family show substantial functional redundancy in isolated neutrophils. In vivo, intratracheal infection of V123 mice with *Staphylococcus aureus* causes an increased bacterial load in the lungs, and infection with *Pseudomonas aeruginosa* results in death (29). In the cutaneous reverse passive Arthus reaction, Vav1$^{-/-}$/Vav3$^{-/-}$ mice fail to develop tissue damage (27). Thus, Vav family GEFs are important for neutrophil-dependent immune functions and inflammatory conditions in vivo.

In this article, we have assessed the relative importance of the P-Rex and Vav families in Rac-dependent neutrophil responses to GPCR stimulation to determine their specific roles and whether they can compensate for each other. We compared the responses of primary neutrophils deficient in either the P-Rex or the Vav family to cells lacking one member of each family. Surprisingly, deficiency in both P-Rex1 and Vav1 caused severe defects in GPCR-dependent neutrophil responses and Rac activation. We propose that either P-Rex1 or Vav1 alone can produce sufficient active Rac for GPCR-dependent cell responses but, under normal circumstances, both contribute, presumably to ensure robust cell responses. In this sense, P-Rex1 and Vav1 cooperate in controlling GPCR-dependent neutrophil responses.

Materials and Methods

Mice

New strains generated for this study are P-Rex1$^{-/-}$/Vav1$^{-/-}$ (P1V1) and P-Rex1$^{-/-}$/Vav3$^{-/-}$ (P1V3) mice, obtained by crossing P-Rex1$^{-/-}$ (P1) (23) and V123 (30) strains (with the latter having been obtained from Martin Turner, Babraham Institute). Both new strains were fertile, albeit with reduced litter sizes, and they appeared healthy when bred and housed under isolator conditions. They were on a mixed 129Ola-C57BL/6-B10Br genetic background and tested against an equivalent wild-type (WT) strain generated from the same original cross.

Previously described strains are: P1, P-Rex1$^{-/-}$/P-Rex2$^{-/-}$ (P12) (31), V123, and Rac2$^{-/-}$ (Rac2), with the latter having been generated by the Williams Laboratory (6) and obtained from Victor Tybulewicz (National Institute for Medical Research, London, U.K.). The P1 mice were on a 129Ola-C57BL/6 genetic background, P12 and Rac2 were on C57BL/6, and V123 was on B10Br. Each strain was tested against control animals of the appropriate background at least until it was established that background had no influence on each cell response tested. All strains were bred and housed under specific opportunistic pathogen-free isolator conditions, except C57BL/6 and P12, which were kept in a shower-in barrier facility.

Neutrophil purification

Unless otherwise stated, all experiments were performed with mature primary neutrophils freshly isolated from the bone marrow of age- and sex-matched mice (at least 8 wk old, usually 8–14 wk old). Purification was performed using a 55/62% Percoll Plus (GE Healthcare, Buckinghamshire, U.K.) gradient and Geyes' solution for erythrocyte lysis as described, with endotoxin-free media throughout (23).

ROS production

ROS production assays were performed in endotoxin-free Dulbecco's PBS, 0.1% glucose, 4 mM $NaHCO_3$ (buffer A; all from Sigma-Aldrich, Dorset, U.K.) in the presence of HRP and luminol (both Sigma-Aldrich), in a temperature-controlled Berthold MicroLumat Plus luminometer (Berthold Technologies, Hertfordshire, U.K.), as described (32). Unprimed neutrophils were stimulated with soluble agonists (3 μM fMLF or 500 nM PMA) immediately after cell preparation, as described (23). Alternatively, neutrophils were primed (or mock-primed) with 1 μg/ml LPS (Sigma-Aldrich) or 500 U/ml TNF (R&D Systems, Minneapolis, MN) at 37˚C for at least 70 or 45 min, respectively, prior to stimulation with 3 μM fMLF.

For assays with *S. aureus* or SRBCs, neutrophils were primed with TNF (1000 U/ml) and GM-CSF (100 ng/ml; PeproTech, Rocky Hill, NJ) at 37˚C for 1 h. Particulate stimuli for ROS assays were prepared as follows: zymosan was IgG-opsonized or serum-opsonized as described (33) and used at a three to five particles per neutrophil ratio. *S. aureus* was grown and serum-opsonized as described (34) and used at a 20 bacteria/neutrophil ratio. Sheep blood (10 μl in Alsever's salt solution [TCS Biosciences, Buckinghamshire, U.K.]) was opsonized with rabbit anti-SRBC IgG (MP Biomedicals, Solon, OH) and resuspended in buffer A.

Chemotaxis

Transwell assays were done essentially as described (35). Cells were either flushed from bone marrow and suspended at 5×10^6 cells/ml in HBSS containing Ca^{2+} and Mg^{2+}, 15 mM HEPES, and 0.25% fatty acid-free BSA (pH 7.4) at 37˚C (buffer B; all reagents endotoxin-free and from Sigma-Aldrich) or purified prior to the assay (23), as specified. Then, 200 μl cells were incubated for 40 min at 37˚C in 3-μm pore polycarbonate filters (Millipore, Billerica, MA) inside ultralow cluster 24-well plates (Sigma-Aldrich) containing 300 μl buffer B, with or without 3 μM fMLF or 3 nM C5a. Cells that migrated through the filter were recovered and neutrophils were identified on the basis of their characteristic forward and side scatter using FACSCalibur (BD Biosciences, Oxford, U.K.) (23). The mean number of neutrophils present in each sample was compared with the mean number originally loaded.

Adhesion and spreading

Neutrophils (1 ml), suspended at 10^6 cells/ml in buffer B, were added to wells containing 13-mm glass coverslips preincubated with 500 μl 20 μg/ml poly(arginyl-glycyl-aspartic acid) (pRGD; Sigma-Aldrich). Cells were incubated with 1.5 μM fMLF at 37˚C for 15 min in 6% CO_2, then fixed with 4% paraformaldehyde/Dulbecco's PBS (pH 7.4) for 15 min and washed twice with PBS. Neutrophils were stained with FITC rat anti-mouse Ly-6G and Ly-6C (Gr1-FITC) Ab (material no. 553126; BD Biosciences, San Diego, CA) and then mounted onto slides. Twelve images from each duplicate coverslip were taken at ×630 magnification using a Zeiss AxioPhot 2 microscope (Zeiss, Jena, Germany) and analyzed for mean number of cells per field of view and mean cell area using Volocity software (PerkinElmer, Waltham, MA).

Expression of Mac-1 integrin

The expression of the integrin Mac-1 ($\alpha_M\beta_2$, CD11b/CD18) on the surface of neutrophils was assessed using total bone marrow cells. Cells were flushed from bone marrow with endotoxin-free HBSS (containing 15 mM HEPES and 0.25% fatty acid-free BSA, but not Ca^{2+} or Mg^{2+} [pH 7.4] at room temperature), then suspended in buffer A at 5×10^7 cells/ml. Two hundred microliters of cells was incubated with or without 20 ng/ml TNF or 50 ng/ml GM-CSF for 30 min at either 0°C or 37°C, then stained with both Gr1-FITC Ab and Cy5 anti-mouse CD11b Ab (catalog no. 19-0112; eBioscience, San Diego, CA) for 20 min at 0°C. Neutrophils were identified from their forward scatter and FITC-stained Gr1 properties using FACSCalibur. The median intensity of Cy5 fluorescence within each sample, determined using FlowJo software (Tree Star, Ashland, OR), was used to assess the expression of Mac-1 on the surface of these neutrophils.

To determine total Mac-1 expression in isolated neutrophils, cells were pretreated with the protease inhibitor diisopropyl fluorophosphate (6 mM) for 10 min at room temperature, and total cell lysates were separated by SDS-PAGE. Western blots for CD11b (catalog no. ab75476; Abcam, Cambridge, U.K.) were performed, and the signal was detected by ECL (GE Healthcare). Coomassie staining of blots was employed to assess loading.

Rac activation

Neutrophils at 1×10^7 cells/ml were preincubated in buffer B, then stimulated for 0–15 s with 10 μM fMLF (Sigma-Aldrich). The reaction was stopped by addition of 5 volumes of ice-cold lysis buffer, and GTP-Rac was isolated from lysates by Pak-CRIB domain pull-down assay as described (23). Two percent of the total lysate was used as a control for Rac1 and Rac2 expression levels. Western blots were performed with monoclonal Rac1 and polyclonal Rac2 Abs (catalog no. 05-389 and 07-604, respectively; both from Upstate Biotechnology [now Millipore]). Blots were then scanned and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analysis

Data were analyzed by one-sample *t* test, with Bonferroni correction; ANOVA with Dunnett post hoc test; or independent *t* test as indicated in figure legends. Statistical significance is designated as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Results

P-Rex and Vav family Rac-GEFs have both been implicated in neutrophil GPCR signaling. In this project, we assessed the roles of the P-Rex and Vav families in GPCR-dependent neutrophil function to determine potential redundancy or cooperation between them. Previous studies have shown that, of the P-Rex family, P-Rex1 is highly expressed in neutrophils, whereas P-Rex2 expression is undetectable, suggesting that P-Rex1 is the only P-Rex family member in these cells (22, 31). All three Vav isoforms are expressed, however Vav2 is 30-fold less abundant than Vav1 and 120-fold less abundant than Vav3 (25); hence, Vav1 and Vav3 are the major isoforms in mouse neutrophils. In this study, we generated mouse strains with homozygous deletions of the predominant isoforms from each family, namely P-Rex1 plus either Vav1 or Vav3 (P1V1 and P1V3). Their neutrophil responses were compared with those from mice lacking the entire P-Rex family (P1 or P12), the entire Vav family (V123), or Rac2.

Western blotting of neutrophil lysates showed that Vav1 expression was unaffected by P-Rex1 deficiency (Supplemental Fig. 1*A*, 1*B*), and P-Rex1 expression was normal in V123 cells (Supplemental Fig. 1*C*). Hence, the loss of one Rac-GEF family was not simply compensated by an overexpression of the other. Similarly, P-Rex1 and Vav1 expression were unaffected by Rac2 deficiency (Supplemental Fig. 1*D*), and the levels of Rac1 and Rac2 were normal in GEF-deficient neutrophils (Supplemental Fig. 1*A–C*). Western blots for Vav3 were unsuccessful due to insufficient sensitivity or specificity of available Vav3 Abs. Expression of P-Rex2 remained undetectable in neutrophils from all strains (data not shown). Furthermore, we assessed the function of neutrophils from P1 and P12 mice in a range of experiments and found them to be comparable (data not shown). Hence, P-Rex2 is unlikely to play a role in neutrophil function, which allowed us to use either P1 or P12 mice to assess the role of the P-Rex family in our experiments.

Blood cell development in P1V1 and P1V3 mice

First, we assessed the impact of GEF deficiency on blood cell development. In peripheral blood, P1V1 mice had twice as many myeloid cells than did WT mice, which could largely be accounted for by elevated levels of granulocytes and eosinophils (Supplemental Fig. 2*A–C*). P1V3 mice showed a similar increase in myeloid cells (mainly monocytes, granulocytes, and basophils) and also exhibited a 60% increase in the number of circulating lymphoid cells (Supplemental Fig. 2*A–C*). For each genotype, the proportion of myeloid cells was near normal in bone marrow, except for P1V1 mice, where they represented 20% of bone marrow WBCs, compared with 12% in WT mice (Supplemental Fig. 2*D*).

P1V1 mice had a trend for decreased total thymocyte numbers (Supplemental Fig. 2*E*), however splenocyte numbers were normal (Supplemental Fig. 2*F*). The number of thymocytes was significantly reduced at the $CD4^+CD8^+$ double-positive and CD4 and CD8 single-positive stages (Supplemental Fig. 2*G*). $CD3^+CD4^+$ and $CD3^+CD8^+$ splenic T cells were also depleted, possibly due to the lack of cells exiting the thymus (Supplemental Fig. 2*H*), whereas spleen B cells (including marginal zone B cells) were normal (not shown). In P1V3 mice, thymic and splenic lymphocyte populations were unaffected (Supplemental Fig. 2*E–H*).

Overall, the effect of P1V1 or P1V3 deletion on bone marrow or circulating blood cells was relatively mild (within 2-fold of WT), whereas T cell development was severely affected in P1V1 (but not in P1V3) mice. The severe defect in P1V1 mice is not surprising, as deletion of Vav1 (but not Vav3) alone causes very comparable defects in lymphocyte development (17), which do not seem to be greatly affected by the additional deletion of P-Rex1.

P-Rex1 and Vav1 cooperate in the regulation of GPCR-dependent ROS production

ROS formation by the NADPH oxidase is a Rac-dependent response. Stimulation of GPCRs elicits ROS formation in freshly isolated neutrophils, and priming with inflammatory agents such as TNF or LPS preserves the ability of the cells to respond to GPCR stimulation up to several hours after preparation (although the extent of priming and total response are variable).

We first studied fMLF-stimulated ROS production in unprimed or TNF-primed neutrophils. Under both conditions, the average total ROS production by P1V1 neutrophils was significantly reduced compared with the response of WT cells (by 60%, $p = 0.005$ [unprimed] and 0.0004 [TNF]; Fig. 1*A*, 1*Bi*, 1*Bii*). In contrast, P1, V123, and P1V3 cells showed only minor, insignificant, defects under the same conditions (Fig. 1*Bi*, 1*Bii*). Despite a large variability between experiments with unprimed V123 cells (the cause of which is unknown but could reflect a hypersensitivity due to the general immunodeficiency of the V123 mice), the average response was comparable with previously published data on $Vav1^{-/-}$/$Vav3^{-/-}$ and V123 neutrophils (10, 28).

In conclusion, neither the entire P-Rex family nor the entire Vav family alone is a major regulator of GPCR-dependent ROS formation in unprimed or TNF-primed cells. However, cells deficient in both P-Rex1 and Vav1 exhibited a greater than additive defect, indicating that these GEFs synergize to generate optimal activity.

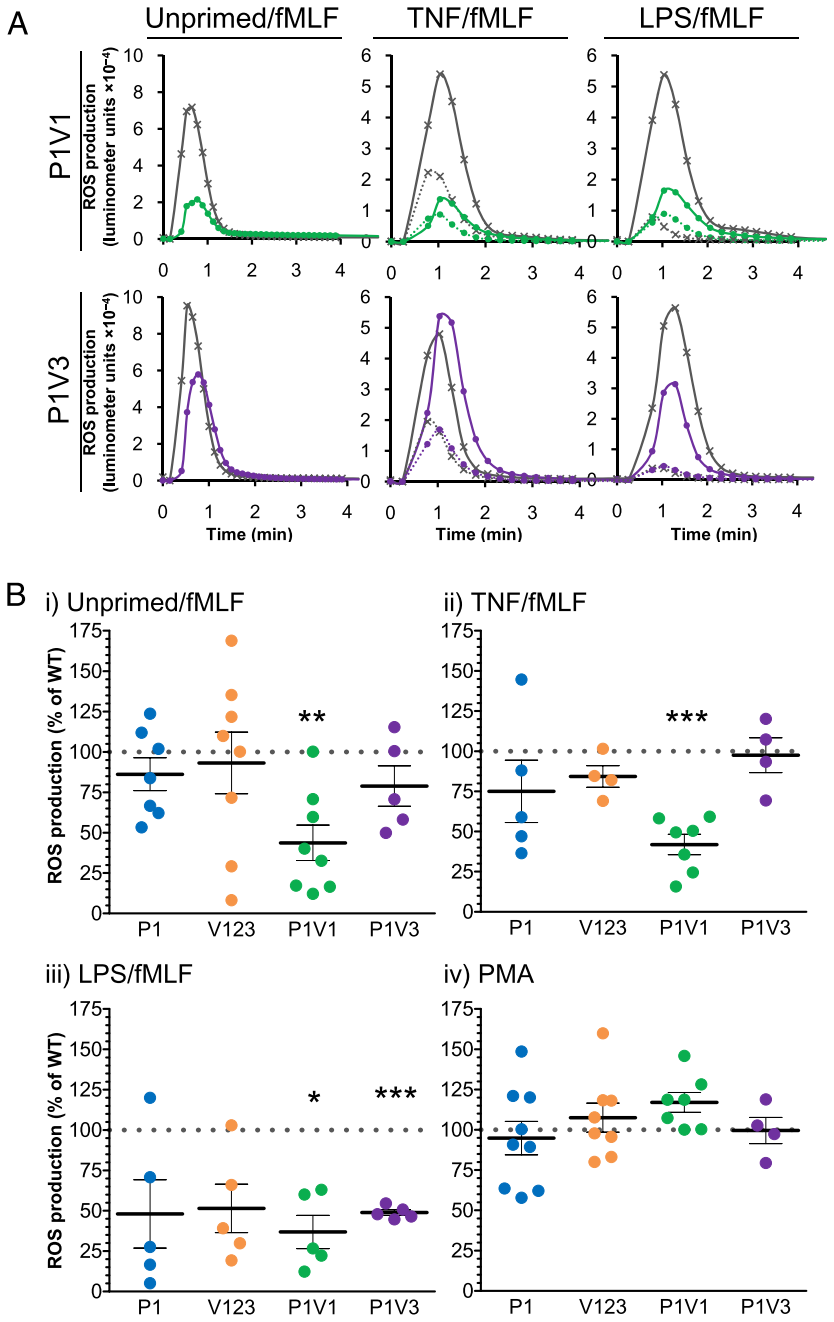

FIGURE 1. P-Rex1 and Vav1 cooperate in fMLF-stimulated ROS formation. *A*, Representative traces of ROS production by P1V1 (*top row*) and P1V3 (*bottom row*) neutrophils (●), recorded for 4 min after stimulation with 3 μM fMLF, and compared with WT cells (×) in each case. Cells were either unprimed or primed with TNF or LPS prior to fMLF stimulation, as indicated. Unprimed cells were tested immediately after preparation, whereas primed or mock-primed cells were assayed after incubation at 37˚C, as detailed in *Materials and Methods*. In the TNF/fMLF and LPS/fMLF traces, primed and mock-primed responses are denoted by solid and dotted lines, respectively. *B*, Total ROS produced by P1, V123, P1V1, and P1V3 neutrophils over 4 min when (*i*) unprimed, (*ii*) TNF primed, or (*iii*) LPS primed prior to fMLF stimulation, or (*iv*) over 9.2 min when PMA stimulated. Total ROS production was determined by the area under the curve for each response and is expressed as a percentage of the corresponding WT response in each case. Each colored dot represents an average of triplicate measurements. Horizontal bars indicate mean values (±SEM) and the dotted line denotes WT response. Differences from WT controls are considered significant at $p < 0.05$ where indicated, as calculated by one-sample *t* tests with Bonferroni correction applied.

Hence, P-Rex1 and Vav1 cooperate in the control of this response. Vav3 is not capable of taking over the role of Vav1 in this cooperation.

When LPS-primed, fMLF-stimulated ROS production in P1V1 neutrophils was reduced by 65% ($p = 0.01$), and by 50% in P1V3 ($p = 3 \times 10^{-5}$), P1, and V123 cells (not statistically significant due to higher variability) (Fig. 1*A*, 1*Biii*). Given the similarity between the responses of each genotype, it can be concluded that LPS priming of fMLF-stimulated ROS production can be mediated by either the P-Rex or the Vav family. Therefore, in contrast to unprimed or TNF-primed GPCR-dependent ROS production, there is no obvious cooperation between the two GEF families in the LPS-primed response.

Receptor-independent ROS production, stimulated by PMA, was normal in cells of all genotypes tested, indicating that the integrity of the NADPH–oxidase complex was not impaired (Fig. 1*Biv*, Supplemental Fig. 3).

The Vav family controls the ROS response to opsonized particles

Opsonized particle-induced ROS production is largely mediated by Fc, complement, and pattern recognition receptors. P-Rex1 deficiency, or double deficiency of P-Rex1 with either Vav1 or Vav3, did not greatly affect opsonized zymosan-induced ROS production; however, the response was totally abolished in V123 neutrophils, regardless of whether zymosan was IgG- or serum-opsonized (Fig. 2, Supplemental Fig. 3). Serum-opsonized *S. aureus*-stimulated ROS responses were normal in P1, P1V1, and P1V3 neutrophils, and IgG-SRBC responses were similarly unaffected in P1 and P1V1 cells (Fig. 2). There was a tendency toward defective ROS production in IgG-opsonized SRBC-stimulated P1V3 neutrophils (Fig. 2), in agreement with a role for Vav3 in FcR-dependent ROS formation (10). As with zymosan stimulation, V123 neutrophils produced very little ROS (90% less than WT) when stimulated with either serum-opsonized *S. aureus* or IgG-

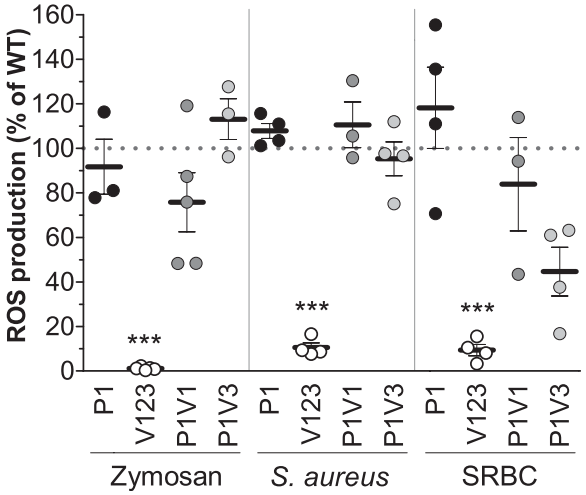

FIGURE 2. The Vav family controls particle-stimulated ROS production in neutrophils. WT, P1, V123, P1V1, and P1V3 neutrophils were primed with TNF and GM-CSF prior to ROS assays, except for zymosan experiments, where cells were unprimed. Neutrophils were then incubated at 37°C with serum- or IgG-opsonized zymosan (3–5 particles/neutrophil ratio), serum-opsonized *S. aureus* (20 bacteria/neutrophil), or IgG-opsonized SRBCs (3 particles/neutrophil) in the presence of luminol and HRP. Light emission was recorded at regular intervals for 44 min (zymosan) or 30 min (*S. aureus* and SRBCs) in a luminometer. Total ROS production was measured by calculating the area under the curve and is expressed as a percentage of the corresponding WT control. Each dot represents average of triplicate measurements. Horizontal black bars indicate mean values (± SEM) and the dotted line represents WT response. Statistical analysis of the difference from the WT response was calculated by one-sample *t* tests with Bonferroni correction applied.

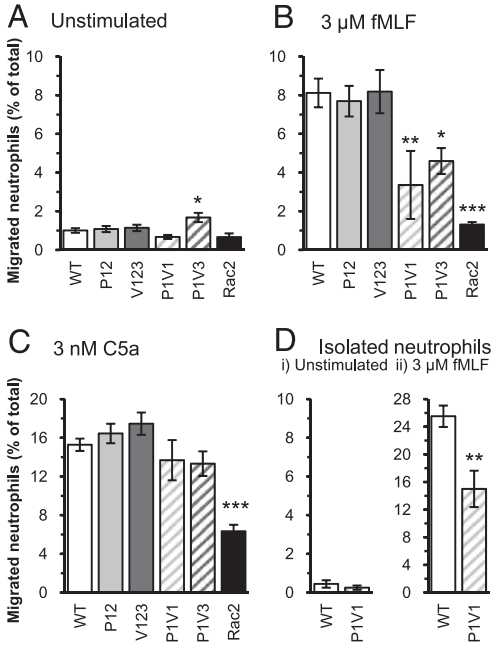

FIGURE 3. P-Rex1 and Vav family GEFs cooperate in fMLF-stimulated neutrophil chemotaxis. *A–C*, Transwell assays with total bone marrow cells, where neutrophil migration through 3-μm filters was measured by flow cytometry. Migration was either (*A*) unstimulated ($n \geq 9$) or stimulated with (*B*) 3 μM fMLF ($n \geq 3$) or (*C*) 3 nM C5a ($n \geq 4$). *D*, Transwell assays with purified WT and P1V1 neutrophils. Migration was either (*i*) unstimulated ($n \geq 3$) or (*ii*) stimulated with 3 μM fMLF ($n = 5$). The mean percentage (±SEM) of neutrophils that migrated within 40 min is shown and data were statistically analyzed either by one-way ANOVA with Dunnett post hoc test, using WT as a control group for each condition (*A–C*), or by independent *t* test (*D*). Data are considered significant at $p < 0.05$.

opsonized SRBCs (Fig. 2), supporting previous findings that the Vav family mediates integrin- and FcR-dependent ROS formation (10, 29). To conclude, in contrast to GPCR-dependent ROS formation, there is no cooperation between the P-Rex and Vav families in the control of particle-induced ROS formation. Furthermore, P-Rex1 is not involved, and the response is entirely dependent on the Vav family.

P-Rex1 cooperates with Vav1 or Vav3 in the control of fMLF-stimulated chemotaxis

We measured chemotaxis using Transwell assays with total bone marrow leukocytes, where neutrophil migration was analyzed by flow cytometry (35). Cells were either unstimulated or stimulated with two different GPCR ligands, 3 μM fMLF or 3 nM C5a (doses within the linear range that elicit robust responses) (Fig. 3*A–C*). In the absence of stimulation, 1% of WT cells migrated through Transwell filters within 40 min, whereas fMLF or C5a stimulation induced chemotaxis of 8 and 15% of WT cells, respectively, within the same time frame.

P1V1 neutrophils showed slightly reduced unstimulated migration, comparable to that of Rac2 cells, whereas P1V3 cells had significantly elevated basal migration (Fig. 3*A*). When stimulated with fMLF, however, chemotaxis of P1V1 neutrophils was significantly lower than WT, regardless of whether cells were purified from total bone marrow prior to the assay or not ($p = 0.009$ and 0.005, respectively; Fig. 3*B*, 3*Dii*). Chemotaxis of P1V3 cells was also significantly lower than WT, by 45% ($p = 0.02$, Fig. 3*B*). Rac2-deficient cells showed an even stronger defect (85% reduction, $p = 8 \times 10^{-7}$), but the migration of P12 and V123 neutrophils was unaffected (Fig. 3*B*), in agreement with previous studies that showed no, or only minor, defects in a variety of chemotaxis assays with isolated P-Rex or Vav family-deficient neutrophils (23, 25, 26). In conclusion, our results suggest that the P-Rex and Vav families cooperate in the regulation of fMLF-dependent neutrophil chemotaxis. Interestingly, P-Rex1 cannot only synergize with Vav1 in this response, but also with Vav3.

An additional level of complexity was observed when measuring chemotaxis in response to C5a. In contrast to fMLF-stimulated chemotaxis, P1V1 and P1V3 cells migrated normally when stimulated with C5a, despite this response being largely Rac2-dependent (Fig. 3*C*). Chemotaxis of P1V1 and P1V3 cells was also unaffected when stimulated with lower concentrations of C5a (data not shown). In conclusion, when neutrophil chemotaxis is induced by two different GPCR ligands, different Rac-GEFs seem to be important, with cooperation between P-Rex1 and Vav1 or Vav3 having been observed in fMLF-stimulated, but not C5a-stimulated, migration.

P-Rex1 and Vav1 cooperate in regulating fMLF-stimulated adhesion of neutrophils

Neutrophils adhere to other cells and to the extracellular matrix via integrins, and this adhesion can be stimulated further by activation of GPCRs. We measured neutrophil adhesion to pRGD, a widely used synthetic integrin ligand. In the absence of further stimulation, neutrophils from all strains adhered to pRGD-coated coverslips normally, although there was a tendency for reduced adhesion in Rac2 cells (Fig. 4*Ai*). Stimulation with fMLF increased the number of adherent WT neutrophils from 8 to 17 per field of view.

fMLF-stimulated adhesion was significantly impaired in P1V1 neutrophils ($p = 0.02$, Fig. 4*Aii*, 4*Aiii*), to the same level as in Rac2 cells (nine per field of view). In contrast, no significant

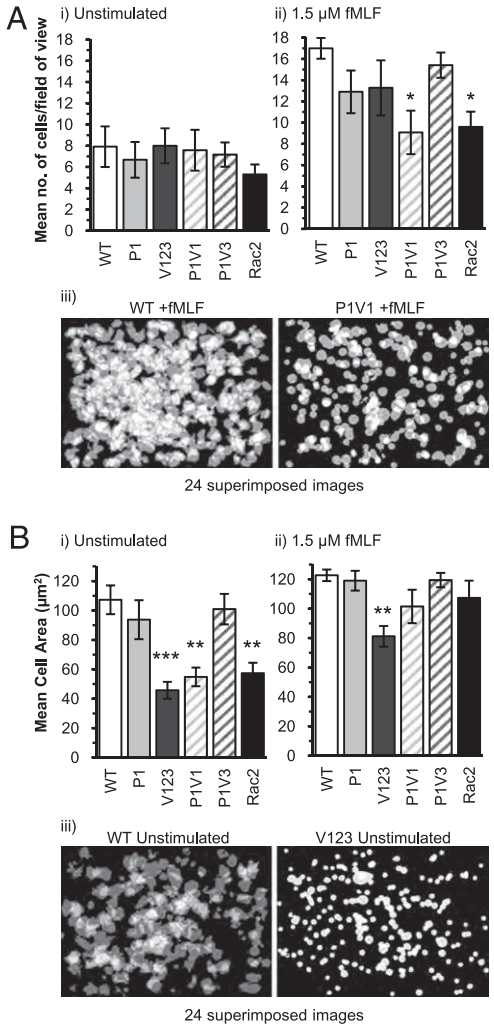

FIGURE 4. P-Rex1 and Vav1 cooperate in the fMLF-stimulated adhesion of neutrophils to pRGD, but cell spreading is Vav family-dependent. *A*, Adhesion. Mean number of (*i*) unstimulated and (*ii*) 1.5 μM fMLF-stimulated neutrophils that adhered per field of view to pRGD-coated coverslips after 15 min (n = 6 independent experiments, ±SEM). *iii*, To illustrate the P1V1 adhesion defect, 24 field-of-view images from representative experiments with fMLF-stimulated WT and P1V1 neutrophils were superimposed. *B*, Spreading. Mean area of (*i*) unstimulated and (*ii*) 1.5 μM fMLF-stimulated neutrophils on pRGD-coated coverslips after 15 min (n = 6, ±SEM). *iii*, The defect in V123 cell spreading is shown by superimposing 24 field-of-view images from representative experiments with unstimulated WT and V123 neutrophils. Image analysis was performed as described in *Materials and Methods*. Statistical significance was assessed by one-way ANOVA with Dunnett post hoc test, using WT as the control group. Where indicated, data are considered significant at $p < 0.05$.

defects were observed in the adhesion of P1V3, P1, or V123 cells (Fig. 4*Aii*). Therefore, whereas neither P-Rex1 nor the Vav family alone is essential, P-Rex1 and Vav1 synergize in the regulation of GPCR-stimulated adhesion to the integrin ligand pRGD. As with fMLF-stimulated ROS formation, Vav3 could not take over the role of Vav1 in this cooperation. We obtained similar results when adhesion to glass was measured (data not shown).

The Vav family controls spreading of neutrophils

Once neutrophils are firmly adhered onto a surface, they undergo Rac-dependent spreading. Unstimulated WT neutrophils spread on pRGD-coated coverslips with a mean surface area of 107 μm^2, as assessed by image analysis of Gr1-FITC–stained cells. P1 and P1V3 cells spread normally (Fig. 4*Bi*); however, the surface area of V123 neutrophils was, on average, 60% smaller than that of WT cells (45 μm^2, $p = 0.0004$, Fig. 4*Bi*, 4*Biii*). Cell area was also significantly reduced in P1V1 and Rac2 neutrophils (to 55 μm^2, $p = 0.002$ and 0.003, respectively; Fig. 4*Bi*). From these data, it can be concluded that P-Rex1 is not involved in spreading on pRGD, and there is no obvious cooperation between the P-Rex and Vav families in this response. Instead, spreading on pRGD appears to be dependent on the Vav family alone (predominantly Vav1), in agreement with previous reports that the Vav family controls integrin-dependent spreading (25).

The addition of 1.5 μM fMLF stimulated an increase in the spreading of cells from all strains. The mean area of P1V1 and Rac2 cells increased to 100 μm^2, compared with 120 μm^2 for WT cells, whereas V123 cells spread significantly less (80 μm^2, $p = 0.005$, Fig. 4*Bii*). Hence, to some extent, fMLF stimulation can overcome the spreading defect caused by Vav deficiency, possibly by stimulating an upregulation of integrins on the cell surface.

P1V1 and Rac2-deficient neutrophils show reduced Mac-1 integrin expression on the cell surface

The defects we observed in fMLF-stimulated adhesion and chemotaxis suggested that P1V1 cells might show changes in their adhesion receptors. To investigate this, we measured the expression of Mac-1 integrin (the major neutrophil adhesion receptor) on the cell surface by flow cytometry. We found that Mac-1 surface expression on freshly isolated P1V1 cells (preserved by incubation at 0°C) was significantly reduced, whereas it was normal in all other genotypes (Fig. 5*A*). Under conditions that allowed integrin trafficking (unprimed cells, incubated at 37°C), Mac-1 surface expression tended to be lower on both P1V1 and Rac2 neutrophils (Fig. 5*B*). This pattern was also evident when integrin upregulation onto the cell surface was actively stimulated by treatment with TNF or GM-CSF. TNF-stimulated P1V1 neutrophils and TNF- or GM-CSF–stimulated Rac2 cells had significantly decreased levels of Mac-1 on the cell surface (Fig. 5*B*).

We performed Western blots for the CD11b subunit of Mac-1 to determine whether the reduction in surface expression on P1V1 and Rac2 neutrophils could be explained by an overall decrease in Mac-1 levels within these cells. CD11b expression was normal in lysates from P1V1 and Rac2 neutrophils, however (Fig. 5*C*). Taken together, our data show that Mac-1 surface expression is sensitive to temperature and priming status in P1V1 and Rac2 neutrophils and that it is reduced under certain conditions, perhaps due to an altered ability of these cells to traffic the receptor. This defect may contribute to the phenotypes of P1V1 and Rac2 cells in integrin-dependent responses such as adhesion and chemotaxis.

GPCR-dependent Rac activation is strongly impaired in P1V1 neutrophils

Our characterization of neutrophil responses suggested that P-Rex1 and Vav1 cooperate in the regulation of fMLF-dependent ROS formation, adhesion, and chemotaxis. We measured fMLF-stimulated Rac activation to determine whether these defects could be explained mechanistically by a reduction in the levels of active Rac1 and Rac2. Indeed, we found that the peak of fMLF-stimulated activation of both Rac1 and Rac2 was diminished by 70% in P1V1 cells (Fig. 6). The extent of this impairment was greater than in P1V3, P1, or V123 neutrophils and appeared to be additive of the defects observed in P1 and V123 cells (Fig. 6*A*). These results show that both P-Rex1 and Vav1 contribute to fMLF-dependent activation of Rac1 and Rac2 in neutrophils and

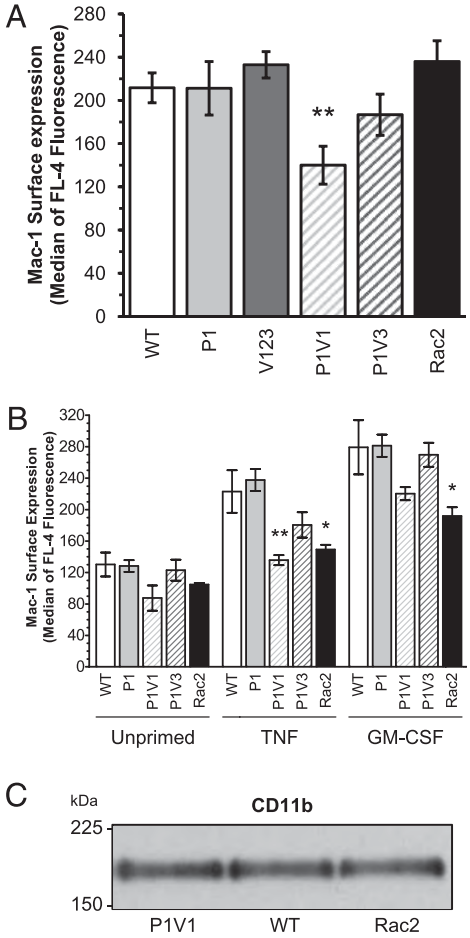

FIGURE 5. Reduced expression of Mac-1 on the surface of P1V1 and Rac2 neutrophils. *A*, Mean expression of Mac-1 on the surface of unprimed neutrophils of the indicated genotypes ($n \geq 6$, ±SEM). Bone marrow cells were incubated for 30 min at 0°C prior to staining with both Gr1-FITC and Cy5-CD11b Abs, and the expression of Mac-1 within the neutrophil population was detected by FACSCalibur analysis. *B*, Mean surface expression of Mac-1 on unprimed, TNF-primed, and GM-CSF-primed neutrophils, incubated for 30 min at 37°C ($n = 4$, ±SEM). For *A* and *B*, statistical significance was determined for each condition by one-way ANOVA with Dunnett post hoc test, using WT as the control group in each case. Data are considered significant at $p < 0.05$. *C*, Western blot for CD11b in WT, P1V1, and Rac2 total neutrophil lysates (from one experiment, representative of two).

that, together, they constitute a major driving force for Rac-dependent signaling downstream of fMLFRs. They also suggest that Vav3 cannot take over the role of Vav1 in this signaling pathway.

Whereas P-Rex1 preferred Rac2 as its in vivo substrate (23), peak activation of Rac1 was reduced by 50%, and Rac2 by 30%, in V123 cells (Fig. 6*A*). This indicates that the Vav family is also capable of activating Rac in response to fMLF, and it may have a preference for Rac1 over Rac2 in vivo. The time course of Rac1 and Rac2 activation was similar in all genotypes, with the peak after 5–10 s of fMLF stimulation, although the response seemed slightly more sustained in V123 cells than in the other strains (Fig. 6*B*). At all time points tested, fMLF-stimulated Rac1 and Rac2 activation was lowest in P1V1 cells (Fig. 6*B*, 6*C*).

The reduced levels of fMLF-stimulated Rac1 and Rac2 activity in P1V1 cells may be sufficient to explain the defects we observed in fMLF-stimulated P1V1 neutrophil responses. For integrin-dependent responses, such as adhesion and chemotaxis, the reduced levels of Mac-1 at the cell surface may contribute further to the defects observed. Mechanistically, we propose that the P-Rex1 or Vav1 pathways alone are capable of producing sufficient active Rac for GPCR-dependent cell responses to proceed but, under normal circumstances, signaling through both pathways occurs, presumably to ensure robust cell responses.

Discussion

The most important finding of our study is that fMLF-dependent ROS formation, chemotaxis, and adhesion are impaired in P1V1 neutrophils but are normal (or near normal) in cells lacking the entire P-Rex family or the entire Vav family. These results suggest that P-Rex1 and Vav1 cooperate in neutrophil GPCR signaling.

Redundancy between enzymes within one family (i.e., of similar domain structure) is common; for example, Src family protein tyrosine kinases (18, 36) and Vav family GEFs (10, 25) play redundant roles within their families in neutrophils. Such redundancy can occur without obvious upregulation of the compensating family member and relies on the mechanisms of regulation being sufficiently similar for one enzyme to take on another's role. Functional redundancy between P-Rex1 and Vav1 is different. Whereas the fMLFR signals through both GEFs and they both activate Rac, separate pathways mediate P-Rex1 and Vav1 activation downstream of the fMLFR: P-Rex1 is targeted by Gβγ and PIP_3 (16), whereas Vav1 is activated by protein tyrosine kinases (17). In WT cells, the fMLFR evidently signals through both pathways to activate Rac, as Vav- and P-Rex1–deficient cells had reduced levels of fMLF-stimulated Rac1 and Rac2 activity, respectively (although the biggest defect in both Rac isoforms was found in P1V1 cells). Such functional redundancy between different pathways downstream of the same receptor is unusual and has been termed cooperation throughout this article.

When either the P-Rex1 or the Vav family pathway is deleted, the other seemingly still provides sufficient active Rac for fMLFR-dependent cell responses to be mounted, as fMLF-stimulated ROS formation, chemotaxis, and adhesion were normal in cells that lacked either the entire P-Rex family or the entire Vav family. We propose that neither pathway alone is essential, but when both P-Rex1 and Vav1 are deleted, the cell can no longer produce sufficient active Rac to mount responses. By operating these pathways in parallel, the cell may safeguard against loss of function. An alternative scenario could be that the P-Rex1 and Vav1 pathways converge, or even synergize, upstream of Rac activation (a more stringent definition of cooperativity), but this seems unlikely given that Rac1 and Rac2 activities are sensitive to the loss of the Vav family or P-Rex1, respectively.

Our concept of cooperation between P-Rex1 and Vav1 does not rule out the possibility of a certain degree of plasticity between their pathways. For example, in P1 cells, where Gβγ and PIP_3-stimulated Rac activation is unavailable, the cell could conceivably sensitize the protein tyrosine kinase/Vav1 pathway, and vice versa. This implies the existence of a sensor for Rac activity and a feedback mechanism that signals the need for more active Rac. The existence of both is purely hypothetical, however.

It would be interesting to investigate whether the P-Rex1 and Vav1 pathways activate separate pools of Rac, or whether the same pool is targeted by both. It seems likely that subtly different subcellular localization of P-Rex1 and Vav1 and/or their differential protein complex formation upon stimulation of the fMLFR may influence which subset of Rac molecules are activated by each GEF within the cell. Our finding that P-Rex1 prefers Rac2 over Rac1 as its in vivo substrate, whereas the Vav family acts mainly on Rac1, suggests that P-Rex1 and Vav1 may indeed activate separate pools of Rac. Preferential P-Rex1 and Vav1 signaling to separate

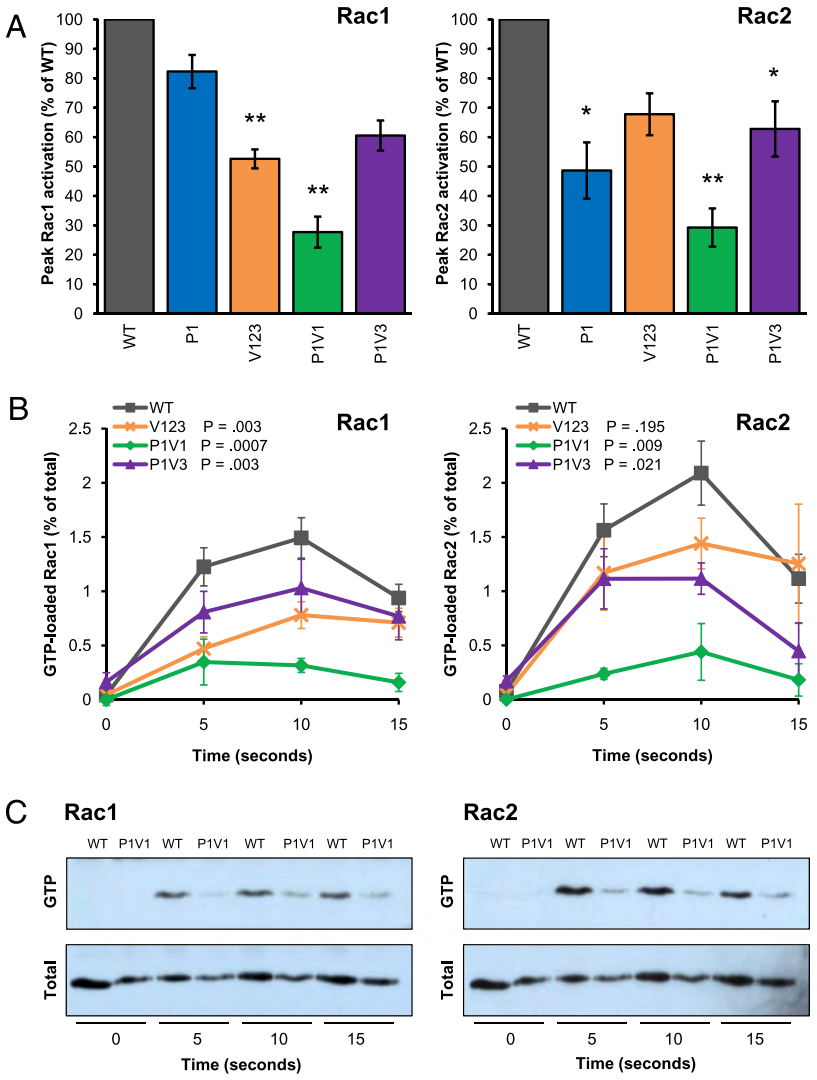

FIGURE 6. fMLF-stimulated activation of Rac1 and Rac2 is strongly deficient in P1V1 neutrophils. WT, P1, V123, P1V1, and P1V3 neutrophils were stimulated with 10 μM fMLF and subjected to Pak-CRIB domain assays to measure Rac1 and Rac2 activation as described in *Materials and Methods*. *A*, Peak Rac1 and Rac2 activation. For each experiment, the peak of Rac1 (*left panel*) or Rac2 (*right panel*) activation was calculated as a percentage of the corresponding WT peak. The average of these values is shown for each genotype ($n \geq 4$, ±SEM). Where indicated, data are significantly different from WT controls at $p < 0.05$, as calculated by one-sample *t* tests with Bonferroni correction applied. *B*, Time course of Rac1 and Rac2 activation. Graphs represent the mean percentage (± SEM) of total Rac1 and Rac2 that were GTP bound after stimulation with fMLF for the indicated times. For clarity, all WT data were pooled. Statistics are one-sample *t* tests on pooled data from 5- to 15-s time points with Bonferroni correction applied. *C*, Representative Western blots for P1V1 cells. The levels of GTP-loaded Rac1 and Rac2 were determined by Western blot (*top panel*) and 2% of the total lysate was blotted as a control for total Rac1 and Rac2 expression (*bottom panel*). Blots are from one experiment representative of four with P1V1 neutrophils.

Rac isoforms could be particularly important for neutrophil responses that require both Rac1 and Rac2, for example, chemotaxis, which involves Rac1 for directionality and Rac2 for movement per se (6, 12), or phagocytosis, during which Rac1 and Rac2 have been shown to have differential membrane localization (37, 38). We are hopeful that, in the future, it might be possible to use fluorescence resonance energy transfer (FRET) imaging technology to visualize the activation of different Rac pools by the P-Rex and Vav families, provided these pools are spatially sufficiently separate.

Previous studies suggested that neither Vav1$^{-/-}$ nor Vav1$^{-/-}$/Vav3$^{-/-}$ neutrophils have defects in fMLF-stimulated Rac activation (25, 26); however, we found that activation of Rac1 and Rac2 in V123 cells was reduced by 50 and 30%, respectively. This discrepancy may reflect the additional deficiency of Vav2 but, as there is low expression of Vav2 in neutrophils (25), it is more likely due to different assay conditions, notably the time points tested. GPCR-dependent Rac activation in neutrophils is rapid and transient (peaks at around 10 s), but the assays available to measure Rac activity in vivo do not allow for extensive time courses. Just as FRET technology is required for visualization of Rac activity in spatial terms, it would also be useful for better temporal resolution and quantification of in vivo Rac activity. Such FRET-based assays are currently available in transfected cell systems (39) but are unfeasible for use with primary neutrophils.

In most instances, P-Rex1 cooperated with Vav1 rather than Vav3, despite Vav3 being 4-fold more abundant than Vav1 in mouse neutrophils (25). The mechanism of this selectivity is unknown, but it may reflect subtle differences in the intracellular localization of Vav1 and Vav3 or their interaction with distinct sets of binding partners. In fMLF-dependent chemotaxis, P-Rex1 could cooperate with either Vav1 or Vav3, despite Rac activation being more severely impaired in P1V1 than in P1V3 cells. This could mean that different Rac pools are activated by Vav1 and Vav3, with the Vav3-dependent pool being more important in chemotaxis than in other responses. Alternatively, Vav3 might play a GEF activity-independent role in chemotaxis. Some GEF activity-independent roles have been described for the Vav family in other cell types; for example, a GEF-dead version of Vav1 can mediate TCR-induced calcium flux and cellular polarization in T cells (40).

Whereas fMLF-dependent responses were controlled by P-Rex1 and Vav1, particle-induced ROS production and cell spreading were solely dependent on the Vav family, and LPS-primed ROS production required both GEF families. This shows that different GEFs couple to different types of receptor, as expected. However, an unexpected level of complexity was observed when comparing chemotaxis in response to different GPCR stimuli. fMLF-dependent migration was impaired in P1V1 and P1V3 neutrophils, but C5a-stimulated migration was normal (although both were dependent on Rac2). Hence, different GEFs control fMLF-dependent

or C5a-dependent chemotaxis. Perhaps, the fMLF and C5a receptors cross-talk with adhesion receptors to a different degree, and this affects which GEFs mediate the response.

Our data show that P-Rex1 and Vav1 together are major regulators of neutrophil fMLFR signaling. However, in addition to the P-Rex and Vav families, which both belong to the Dbl superfamily, neutrophils also express DOCK2, a Rac-GEF from the structurally unrelated DOCK superfamily (41). The mechanisms regulating DOCK2 activity in neutrophils are unknown, but DOCK2 membrane translocation (which is required for function) is known to depend on PIP_3 and phosphatidic acid formation (42, 43). GPCR-stimulated DOCK2$^{-/-}$ neutrophils can sense chemoattractant gradients but migrate with reduced speed, lack the polarized accumulation of F-actin and PIP_3 at the leading edge, and show substantially reduced Rac1 and Rac2 activity (42). Therefore, DOCK2 is also an important mediator of GPCR-dependent neutrophil responses. DOCK2$^{-/-}$ cells differ from P-Rex and/or Vav-deficient neutrophils in several aspects. PMA-dependent ROS formation is impaired in DOCK2$^{-/-}$ cells, suggesting that their NADPH–oxidase complex might not assemble as a fully functioning enzyme (42). Furthermore, C5a-stimulated migration is strongly impaired in DOCK2$^{-/-}$ neutrophils (42), but not in P-Rex and/or Vav family deficient cells. These differences suggest that P-Rex1/Vav1 and DOCK2 play nonredundant roles in neutrophil GPCR signaling, but it is currently unknown whether DOCK2 functions in parallel, in series, or independently of P-Rex and Vav. It would be interesting to investigate whether DOCK2 can cooperate with other neutrophil GEFs in GPCR signaling in a manner similar to P-Rex1 and Vav1.

We sought to define the GPCR-dependent functional roles of P-Rex and Vav family GEFs in neutrophils. To our knowledge, this is the first direct comparison of different GEF families in primary cells and the first time the possibility of redundancy between families has been addressed. Our major finding was that P-Rex1 and Vav1 cooperate in controlling several GPCR-dependent neutrophil responses. We have also presented evidence that this cooperation is specific to GPCR signaling, as particle-induced neutrophil responses were entirely dependent on the Vav family, and LPS signaling required both GEF families. Hence, our study has revealed unexpected levels of complexity in neutrophil signaling.

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Disclosures

The authors have no financial conflicts of interest.

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